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User's Guide for Conducting Life-Cycle Chronic Toxicity Tests with Fathead Minnows (*Pimephales promelas*)

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USER'S GUIDE FOR CONDUCTING LIFE-CYCLE CHRONIC TOXICITY TESTS WITH FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

by

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FOREWORD

The original chronic bioassay procedures for fathead minnows were compiled in 1971 by John Eaton for the Committee on Aquatic Bioassays at the U.S. Environmental Research Laboratory-Duluth. These methods were then published in Standard Methods for the Examination of Water and Wastewater (1).

The current revised procedures for 1981 represent an updated and reorganized version of the old methods. These new procedures are based on recent evaluations of toxicity test results and methods used to conduct life-cycle chronic tests and early life stage tests with fathead minnows.

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ABSTRACT

This paper represents a revised procedural guide for conducting life-cycle chronic toxicity tests with fathead minnow (Pimephales promelas). These new procedures are based on recent evaluations of published toxicity tests and methods used by aquatic toxicologists to conduct life-cycle chronic tests and early life stage tests with fathead minnows. These published papers are referenced in the appropriate place throughout the text of this report. If more detailed information on test apparatus or specific biological and chemical methods is desired, the reader is encouraged to study the reference material.

All routine methods not covered in this procedure (e.g., physical and chemical determinations, handling of fish) should be followed as described in Standard Methods for the Examination of Water and Wastewater (1).

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CHAPTER 1

PHYSICAL SYSTEM

1.1 Diluter

Intermittent-flow proportional diluters (2,3) or continuous-flow serial diluters (4) should be employed. The operation of the diluter should be checked daily, either directly or through measurement of toxicant concentrations. A minimum of five toxicant concentrations with a dilution factor not greater than 0.50 and one control should be used for each test. An automatically triggered emergency aeration and alarm system should be installed to alert staff in case of diluter, temperature control or water supply failure.

1.2 Toxicant Mixing and Flow Splitting

If a proportional diluter is used, a container to promote mixing of toxicant and diluent water should be used between the diluter and test tanks for each concentration (5). Separate flow splitter delivery tubes should run from this container to each replicate larval and adult tank. If a continuous-flow serial diluter is used, additional mixing containers are not needed but separate flow splitter delivery tubes must run from the diluter to all test tanks. Delivery tubes are allocated to tanks by stratified random assignment. Flow splitting accuracy must be within 10% and should be checked periodically to see that the intended amounts of test water are going to each tank.

1.3 Test Tanks

All test tanks should be made of either glass or stainless steel with glass ends. Two arrangements of test tanks are recommended.

1.3.1 Arrangement A

Under this arrangement, duplicate adult spawning tanks measuring 30.5 x 30.5 x 91.4 cm long are used. A 30.5 cm square portion at the upper end of each tank is screened off for larval exposures. Each larval section is divided in half so that there are two larval growth chambers for each adult spawning tank (6). Larval chambers should be designed with glass bottoms and drains that allow water to be drawn down to 3 cm when the chamber is lifted out of the larval section for photographic growth measurements (see Section 3.4).

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Test water must be delivered separately to each adult tank and larval section, with one-third of the water volume going to the latter. Test water delivered to each larval section must also be split evenly to each growth chamber. Test water depth in adult tanks and larval chambers should be a minimum of 15 cm.

1.3.2 Arrangement B

Duplicate adult spawning tanks measuring 30.5 x 30.5 x 61 cm long and separate larval tanks are used in this arrangement. Each larval tank should be a minimum of 28,373 cubic cm and should be divided in half to form two larval growth chambers for each adult spawning tank (7). Larval chambers are designed and test water divided as previously described in Section 1.3.1. Larval tanks can be conveniently located directly above spawning tanks containing test solutions of the same concentrations so they can be drained directly into the spawning tank.

1.4 Embryo Incubation Cups

Embryo incubation cups should be made from 120 ml glass jars (5.1 cm OD) with the bottoms cut off and replaced with stainless steel or nylon screen (40 meshes per 2.54 cm). Cups should either be oscillated vertically (2.5-4.0 cm) in the test water by means of a rocker arm apparatus driven by a 2 rpm motor (8) or placed in separate chambers with self-starting siphons. Both methods will produce a frequent flow of test water around the embryos during the 4-5 day incubation period. Cups should not be hung in tanks containing juvenile or adult test fish, unless each cup is designed with an additional double bottom stainless steel screen ($\geq$ 10 mesh per 2.54 cm) to prevent fish from sucking embryos through the 40 mesh screen bottom.

1.5 Spawning Substrates

Fathead minnows deposit their adhesive eggs on the underside of submerged objects. The following types of laboratory spawning substrates for fathead minnows have been tested and are recommended for use in culture units and life-cycle chronic tests (9).

1.5.1 Cement

Cement drain tiles (7.6 to 10.2 cm ID) can be used for spawning substrates. Tiles must be cut into 7 to 10 cm long sections and then halved lengthwise and inverted to form a semicircular arch.

1.5.2 Stainless Steel

Stainless steel (#316, 16-18 gauge) cut and bent into the same shape and size as described above with a thin layer of renewable quartz sand coated on the

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underside can also be used as a spawning substrate. Embryos adhering to the above substrates are rolled off with a gentle circular motion of the finger while pressing on them lightly (10).

1.6 Diluent Water

The water used should be from a well or spring, if at all possible, or alternatively from a surface water source. Only as a last resort should dechlorinated water from a municipal water supply be used. If there is any chance the water supply could be contaminated with fish pathogens, the water should be passed through an ultraviolet or similar sterilizer immediately before it enters the test system (6).

1.7 Flow Rate

Flow rates to each adult tank or larval chamber should be equal to at least 6-10 tank or chamber water volumes per 24 hours. Flow rates must be great enough so that dissolved oxygen does not drop below 75% of saturation (11) or toxicant concentrations to drop by more than 20% when fish are in the test tanks. Flow rates can be increased above those specified to maintain proper dissolved oxygen or toxicant concentrations.

1.8 Aeration

Diluent water should be aerated vigorously (with oil-free air) or passed over a screen column with a recirculating pump before flowing through the diluter. Aeration of diluent water will eliminate supersaturation of dissolved gases and also insure that dissolved oxygen concentrations will be at or near saturation (90-100%). However, the test tanks and chambers themselves should not be aerated.

1.9 Test Water Temperature

Test water temperature should not deviate from 25° C by more than 2° C and should not remain outside the range of 24 to 26° C for more than 48 hours at a time (12). Temperature should be recorded continuously.

1.10 Light

The lights used should simulate the wavelength spectra of sunlight as nearly as possible. A combination of Durotest (Optima FS) fluorescent tubes manufactured by Duro-Test Corporation, Secaucus, NJ 07094, and wide spectrum Grow-Lux fluorescent tubes manufactured by Sylvania Lighting Center, Danvers, MA 09123 has proven satisfactory (13). Light intensities at the water surface during tests have ranged from 10-100 lumens.

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1.11 Photoperiod

In order to maintain standard periods of light, photoperiods (Table 1-1) should simulate the dawn-to-dusk times of Evansville, Indiana (representing average light conditions for the middle of the continental United States). Adjustments in day-length are to be made on the first and fifteenth day of every Evansville test month (14). The table is arranged so that adjustments need be made only in the dusk times. Regardless of the actual date that the experiment is started, the amount of light used should be the same as the Evansville test photoperiod of December 1. To illustrate this point, an experiment started with 24-hour-old embryos in Duluth, Minnesota, on August 28 (actual date), would require use of a December 1 Evansville test photoperiod, and the lights could go on anytime on that day as long as they remained on for 10 hours and 45 minutes. Fifteen days later (September 12 actual date, December 15 Evansville test date) the day-length would be changed to 10 hours and 30 minutes. Gradual changes in light intensity at dawn and dusk (15) may be included within the photoperiods if desired, but should not last for more than 30 minutes from full-on to full-off and vice versa.

1.12 Cleaning

All adult tanks and larval chambers (after larvae swim-up) must be siphoned at least three times a week and brushed when algal or fungal growth becomes noticeable. Siphoning should be done just before the last feeding of the day. Incubation cup screen bottoms should also be brushed periodically if they become clogged.

Siphoning can be done safely with either a large pipette (50 ml) fitted with a squeeze bulb or a glass tube and siphon hose leading to a white pan. Fish which are siphoned accidentally can be observed easily in the pipette or white pan and returned carefully without harm to the chamber.

1.13 Disturbance

Adults and larvae should be shielded from disturbances such as people walking past the tanks or extraneous lights that might alter the intended photoperiod.

1.14 Construction Materials

Construction materials which contact the diluent water should not contain leachable substances and should not absorb significant amounts of substances from the water. Stainless steel is the preferred construction material. Glass significantly absorbs some trace organics. Rubber must not be used. Plastic containing fillers, additives, stabilizers, plasticizers, etc., must not be used. Teflon, nylon, and their equivalents are not known to contain leachable materials, nor do they absorb significant amounts of test substances. All batches of neoprene stoppers should be checked for toxicity

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TABLE 1-1

TEST PHOTOPERIOD (EVANSVILLE, INDIANA) FOR
FATHEAD MINNOW LIFE-CYCLE CHRONIC TOXICITY TESTS

Dawn to Dusk Time	Date		Day-length (hour and minute)	
6:00 - 4:45	Dec.	1	10:45	Day one of life-cycle
6:00 - 4:30		15	10:30	chronic test
6:00 - 4:30	Jan.	1	10:30	
6:00 - 4:45		15	10:45	
6:00 - 5:15	Feb.	1	11:15	5-month pre-spawning
6:00 - 5:45		15	11:45	growth period
6:00 - 6:15	Mar.	1	12:15	
6:00 - 7:00		15	13:00	
6:00 - 7:30	Apr.	1	13:30	
6:00 - 8:15		15	14:15	
6:00 - 8:45	May	1	14:45	
6:00 - 9:15		15	15:15	
6:00 - 9:30	June	1	15:30	4-month spawning
6:00 - 9:45		15	15:45	period
6:00 - 9:45	July	1	15:45	
6:00 - 9:30		15	15:30	
6:00 - 9:00	Aug.	1	15:00	
6:00 - 8:30		15	14:30	
6:00 - 8:00	Sept.	1	14:00	
6:00 - 7:30		15	13:30	
6:00 - 6:45	Oct.	1	12:45	Post-spawning period
6:00 - 6:15		15	12:15	
6:00 - 5:30	Nov.	1	11:30	
6:00 - 5:00		15	11:00	

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prior to their use in the diluter and exposure chambers. Recent static tests at the U.S. EPA, Environmental Research Laboratory, Duluth have shown that certain lots of neoprene stoppers are acutely toxic to fathead minnow larvae (S. J. Broderius, personal communication, U.S. Environmental Protection Agency, Duluth, MN 55804).

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CHAPTER 2

CHEMICAL SYSTEM

2.1 Preparing a Stock Solution

Distilled or diluent water should be used in making-up the test stock solutions. The recent development of several chemical saturators for use with hydrophobic chemicals has eliminated the need to use carrier solvents with most test chemicals (16,17,18,19).

If carrier solvents other than water are absolutely necessary, reagent grade or better should be used, but amounts must be kept to a minimum. Triethylene glycol (TEG) and dimethyl formamide (DMF) are preferred, but methanol, ethanol or acetone can also be used. The calculated solvent concentration to which any test organisms are exposed must never exceed 0.1 ml/liter.

When a carrier is used, use two sets of duplicate controls. One set should contain no solvent and one set should contain the highest concentration of solvent to which any organisms in the test are exposed.

2.2 Measurement of Toxicant Concentration

As a minimum, the concentration of toxicant must be measured in one tank at each toxicant concentration every week, alternating between duplicate tanks at each concentration from week to week. Water samples should be taken about midway between the top and bottom and the sides of the tank and should not include any surface scum or material stirred up from the bottom or sides of the tank.

2.3 Measurement of Other Variables

Dissolved oxygen must be measured at each concentration at least once a week. In alternating weeks, the opposite tank at each concentration should be measured for dissolved oxygen.

A control and one test concentration must be analyzed at least weekly for pH, alkalinity, hardness, and conductance to show the variability in the test water. If any of these characteristics is affected by the toxicant, that characteristic must be measured at each concentration at least once a week and alternated between duplicate tanks.

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At a minimum, the test water must be analyzed twice during the test period for calcium, magnesium, sodium, potassium, chloride, sulfate, total solids, and total dissolved solids.

2.4 Residue Analysis

Mature fish, and possibly eggs, larvae, and juveniles, obtained from the test, must be analyzed for toxicant residues. Since fathead minnows usually are consumed as whole organisms by predators, residues should be determined for whole bodies rather than individual tissues.

2.5 Methods

Methods described in Methods for Chemical Analysis of Water and Wastes (20) and Manual of Analytical Methods for the Analysis of Pesticides in Human and Environmental Samples (21) should be used for chemical analysis when possible. Accuracy should be measured using the method of known additions for all analytical methods for toxicants. Reference samples should be analyzed periodically for each analytical method.

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CHAPTER 3

BIOLOGICAL SYSTEM

3.1 Source of Test Fish

Sufficient numbers of embryos are transported easily from a brood stock culture unit to other laboratories or field sites to initiate a life-cycle chronic test. These embryos usually are shipped in well-oxygenated water in insulated containers.

Laboratory brood stock culture units can be started by obtaining embryos from a well-established culture unit such as is maintained at the Environmental Research Laboratory in Duluth, MN. This laboratory periodically mixes their brood stock with healthy wild minnows to eliminate the risk of developing a homogeneous strain.

At 25° C and a constant 16-hour day-light photoperiod, fish fed unrestricted quantities of live brine shrimp nauplii and frozen adult brine shrimp will mature in 5-6 months. With proper care and maintenance these adult fish will produce large numbers of embryos for 6-8 months.

Paired spawning, as opposed to group spawning, eliminates fighting and competition. This method is recommended for use in brood stock culture units and life-cycle chronic tests. Experiments conducted by Benoit and Holcombe (7) have demonstrated the successful use of paired spawning in individual, screened, spawning chambers. These investigators correctly determined the sex of 40 pairs of mature minnows. Results of their test showed that 38 out of 40 pairs of adults spawned repeatedly. When the U.S. Environmental Research Laboratory-Duluth, MN, began to use this approach, the number of embryos produced in the stock culture units almost doubled. Each culture unit consists of one tank measuring 30.5 cm x 30.5 cm x 61 cm long with a water depth of 18 cm and four individual spawning chambers (15.2 x 30.5 cm) formed by stainless steel screen dividers (5 mesh, 0.89 mm wire).

3.2 Preliminary Tests

Selection of the test concentration for the life-cycle exposure should be determined on the basis of the results of a 6-10 day range-finding test and a 96-hour toxicity test (22) with either larval or juvenile fathead minnows. Unless the test chemical is extremely toxic, the highest test concentration selected for the chronic exposure should usually be no less than the 96-hour LC20 and no greater than the 96-hour LC50. All fish used in preliminary and life-cycle tests should be from the same adult stock.

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3.3 Embryo Exposure (4-5 days)

The life-cycle chronic toxicity test must begin with embryos from at least three separate spawnings that are $\leq$ 24-hours-old and have soaked in dilution water for at least 2 hours. Prior to the start of the test all embryos also must be viewed carefully with a dissecting scope or magnifying viewer to remove empty shells and opaque, or abnormal appearing embryos. If less than 50 percent of the embryos from a substrate appear to be healthy and fertile, all embryos from that substrate should be discarded. Embryos stuck together in clumps of four or more are either manually separated or discarded.

The test is started by impartially distributing 50 embryos ($\leq$ 24-hour-old) to each of the four replicate larval growth chambers using the following suggested method: 10 embryos are impartially selected and transferred with a large bore eye dropper to successive incubation cups which are standing in dilution water. This process is repeated until 50 embryos are in each cup. The incubation cups are then distributed by stratified random assignment to each replicate larval chamber.

Dead embryos usually will turn opaque and must be counted and removed each day until hatching is complete. Live fungused embryos also must be removed daily, and are subtracted from the original total when calculating percentage hatch. Embryos found floating in the incubation cup can be submerged by gently proding with a glass rod or by gently swirling the cup in the test water.

Upon completion of hatching, the total number of larvae, in each replicate, including those dead or deformed, are counted. Dead or deformed larvae are subtracted from the total in determining the number of normal larvae at hatch. Time to complete hatch in each cup is recorded to the nearest day.

3.4 Larval-Juvenile Exposure (8 weeks)

After hatching, each group of larvae is randomly reduced to 25, and released in replicate larval growth chambers. All live fish that are lethargic or deformed must also be included in the random selection. Another option to the above method would be to impartially reduce the embryos to 25 before hatching and release all of the larvae into the growth chamber after hatching.

Larvae should be fed within two days after hatching. Each group must be fed live, newly hatched, brine shrimp nauplii three times a day at least four hours apart (or two times a day about six hours apart) during the first 3 weeks after hatching (23). Beginning four weeks after hatching, all fish should be fed frozen adult brine shrimp at least twice a day (7). The amount of food provided to each chamber must be proportional to the number of fish in the chamber. Each batch of brine shrimp eggs and adults should be analyzed for pesticides.

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Survival should be determined in each replicate growth chamber at least once a week. Larvae which die during the first two weeks after hatching generally deteriorate so rapidly that they cannot be observed easily in the test chambers. Therefore, survival during this period is determined by counting the number of live fish.

Record the number of abnormal fish at four and eight weeks after hatching and measure total lengths of all fish in each replicate growth chamber using photographic methods of McKim and Benoit (24). Each glass growth chamber containing fish is removed from the larval section of the tank and water drained to a depth of 3 cm. The chamber then is transferred to a viewing box having fluorescent lights under a squared millimeter grid of adequate size to accommodate the growth chamber. Photos then are taken of the fish over the grid and enlarged into 20 x 25 cm prints. The length of each fish is determined subsequently determined by comparing it to the grid. This method allows growth measurements to be made without handling or removing test fish from the water, and accuracy is within 1% of the actual fish length.

3.5 Juvenile-Adult Exposure (32-40 weeks)

Eight weeks after hatching, all fish in each growth chamber must be transferred to the adult spawning tank of the same concentration and randomly reduced to 25 fish per tank. Deformed individuals also must be included in the random selection. If necessary, in order to obtain 25 fish in each spawning tank, several fish may be selected randomly for transfer from one tank to another of the same concentration. Record the length and weight of all fish discarded from each spawning tank.

Four spawning substrates also should be placed in each spawning tank at this time. Substrates should be separated widely to reduce interaction and situated so that the underside of the substrates can be viewed from the end of the spawning tanks.

Continue routine feeding and cleaning and checking mortality throughout the juvenile-adult exposure. Handle disease outbreaks according to their nature, with all experimental and control tanks receiving the same treatment whether or not there seems to be sick fish in all of them. The frequency of treatment should be held to a minimum (22).

When secondary sexual characteristics are well-developed and males begin to establish territories (approximately 20-24 weeks after hatch), separate males, females and undeveloped fish in each spawning tank (7). Sex can be determined on maturing fish by viewing each group in a glass aquarium with a lighted background. Mature males will exhibit tubercles, pads and body color (25,26); mature females will exhibit extended, transparent anal canals (urogenital papilla) as described by Flickinger (27).

At this time, four individual spawning chambers (see Section 3.1) are formed in each spawning tank and one spawning substrate placed in each chamber.

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Four males and four females from each spawning tank then are chosen randomly and assigned to spawning chambers. If necessary, in order to establish four mature pairs in each spawning tank, several fish should be selected randomly for transfer from one tank to another of the same concentration. However, if there are not enough mature fish in either spawning tank, these fish must be placed back into their respective tanks, without screen dividers, and observed daily for sexual development and/or spawning activity.

All surplus adults and undeveloped fish are weighed, measured and cut open for positive sexual identification. Males and females that are readily distinguishable from one another because of their external characteristics should be selected initially for determining how to differentiate between testes and ovaries. The gonads of both sexes will be located just ventral to the kidneys.

Substrates are checked for spawnings daily, including weekends, (preferably during the Evansville afternoon hours) and embryos removed as described in Section 1.5. Substrates must be replaced immediately after embryos are removed. All embryos produced in each spawning are counted and recorded separately for each pair.

The adult exposure should be terminated when, during the decreasing day-length photoperiod, a one-week period passes in which no spawning occurs in any of the tanks. Record total lengths, weights and sex of parental fish. At this time the gonads of most parental fish will have begun to regress from the spawning condition, and both external and internal differences between the sexes will be less distinct. The ovaries are generally larger than testes and will appear transparent, but may still contain some coarsely granular yellow pigment. The testes will be quite slender with very fine granular strands and may be slightly milky when squeezed.

3.6 Second Generation Embryo Exposure (4-5 days)

Fifty embryos from the first five spawnings ($\geq$50 embryos) and every third spawning ($\geq$50 embryos) thereafter from each pair are selected impartially and transferred to incubation cups for hatch. Those embryos not selected for incubation are discarded.

Test procedures used during the embryo incubation and hatching of offspring are described in the last two paragraphs of Section 3.3.

3.7 Second Generation Larval-Juvenile Exposure (4-8 weeks)

Two groups of 25 larvae produced from different pairs in each spawning tank are released in replicate growth chambers for eight week exposures. Selection of each group should be from early spawnings so that the chambers will be available for additional exposures of fish, if desired.

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Test procedures used during the selection and exposure of offspring are described in Section 3.4.

Each group of second generation fish is terminated eight weeks after hatching. Individual fish are blotted, weighed, and measured before discarding or freezing.

If the test is continued beyond the four week period or fish are transferred to clean water for residue half-life studies, individual live fish can be blotted through a small net and weighed by water displacement. Length can be determined photographically on live fish as described in Section 3.4.

3.8 Additional Exposures and Special Examinations

Important information on hatchability and larval survival can be gained by transferring, immediately after spawning, embryos from the control tanks to tanks having toxicant concentrations in which spawning is known to be reduced or absent or to tanks in which an effect is seen on survival of embryos or larvae. Information also can be gained by transferring embryos from these high toxicant concentrations to the control tanks.

Fish and embryos obtained from the test can be used for physiological, biochemical, histological, and other tests that may indicate certain toxicant related effects. Egg adhesiveness can also be evaluated with the use of egg traps placed under selected spawning substrates (7).

Extended life-cycle chronic tests may be conducted through several generations by using the test procedures described in sections 3.3 through 3.7.

3.9 Biological Data Recorded

3.9.1 Embryo Exposure

Days to complete hatching; total number of embryos hatched; and number of normal larvae at hatching in each incubation cup.

3.9.2 Larval-Juvenile Exposure

Survival, deformities, and growth of fish at the end of the 4 and 8 week exposure periods in each replicate growth chamber.

3.9.3 Juvenile-Adult Exposure

Survival and deformities of fish at the time of selection for paired spawning; growth and sex of discarded fish not selected for spawning; and survival, growth, and deformities of male and female fish at the end of the

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spawning test period in each duplicate spawning tank. In addition to the foregoing data, the number of embryos per spawn and the total number of spawnings by each pair of adults in individual spawning chambers also are recorded.

3.10. Data Analysis and Evaluation

Test results can be analyzed statistically by standard techniques. A simple one-way analysis of variance may be used initially to assess whether significant ($P \geq 0.05$) differences have been found (28).

If differences exist, the investigator can then determine whether responses for a given concentration are significantly ($P \geq 0.05$) different from responses for the control. Two particularly appropriate methods for this type of evaluation are Dunnett's test (29) and William's test (30,31). Duncan's new multiple-range test also can be used to analyze statistically differences between all treatments (32).

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